Original Paper

Salt-Inducible Kinase 1 (SIK1) is Induced by Alcohol and Suppresses Microglia Inflammation via NF- κ B Signaling

Yu Zhang^a Weida Gao^b Kongbin Yang^c Haiquan Tao^b Haicheng Yang^b^aDepartment of Neurology, The Fourth Affiliated Hospital of Harbin Medical University, Harbin,^bDepartment of Neurosurgery, The Second Affiliated Hospital of Harbin Medical University, Harbin,^cDepartment of Neurosurgery, The First Affiliated Hospital of Harbin Medical University, Harbin, China**Key Words**Salt-Inducible Kinase 1 • Alcohol • Brain Inflammation • NF- κ B Signaling**Abstract**

Background/Aims: Alcohol consumption has been shown to cause neuroinflammation and increase a variety of immune-related signaling processes. Microglia are a crucial part of alcohol-induced neuroinflammation and undergo apoptosis. Even though the importance of these inflammatory processes in the effects of alcohol-related neurodegeneration have been established, the mechanism of alcohol-induced microglia apoptosis is unknown. In prior research, we discovered that alcohol increases expression of salt-inducible kinase 1 (*SIK1*) in rodent brain tissue. In this study, we sought to determine what role *SIK1* expression plays in alcohol-induced neuroinflammation as well as whether and by what mechanism it regulates microglia apoptosis. **Methods:** Adult C57BL/6 mice were divided into four groups and for 3 weeks treated with either 0%, 5%, 10%, or 15% alcohol during 3 hour periods. The mice were sacrificed and their brains excised for analysis. Additionally, primary microglia were isolated from neonatal mice. *SIK1* expression in alcohol-treated brain tissue and microglia was analyzed via RT-PCR and western blotting. TUNEL staining, caspase-3, and caspase-9 activity assays were performed to evaluate microglial apoptosis. Cell fluorescence staining and NF- κ B luciferase activity assays were used to evaluate the effects of *SIK1* expression on the NF- κ B signaling pathway. **Results:** *SIK1* expression was increased in the brains of mice that consumed alcohol, and this effect was seen in mouse primary microglia. *SIK1* knockdown in microglia increased alcohol-induced apoptosis in these cells. Furthermore, *SIK1* reduced NF- κ B signaling pathway factors, and *SIK1* knockdown in microglia promoted alcohol-induced NF- κ B activity. TUNEL staining, caspase-3, and caspase-9 activity assays consistently revealed that alcohol-induced microglial apoptosis was inhibited by depletion of p65. Finally, we determined that NF- κ B signaling is required for alcohol-induced, *SIK1*-mediated apoptosis in microglia. **Conclusion:** This study establishes for the first time not only that *SIK1* is crucial to

Yu Zhang, Weida Gao and Kongbin Yang contributed equally to this work.

Haiquan Tao and
Haicheng YangDepartment of Neurosurgery, The Second Affiliated Hospital of Harbin Medical
University, 148 Baojian Road, 150081 Harbin, (China)
Tel. 86-0451-86605222, E-Mail taohaiquan7824@163.com/yanghaicheng999@sina.com

regulating alcohol-induced microglial apoptosis, but also that the NF- κ B signaling pathway is required for its activity. Overall, our results help elucidate mechanisms of alcohol-induced neuroinflammation.

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Introduction

Microglial activation is a hallmark of many neurodegenerative processes, including those induced by acute alcohol use [1, 2]. Changes in microglia activity and increases in pro-inflammatory cytokines are thought to both result from and contribute to alcohol pathologies [3, 4]. The effects of alcohol exposure on the immune system are complex, and adding to this complexity is the fact that microglia can function in different contexts as either neuroprotective or cytotoxic agents [5]. On the one hand, microglia exacerbate alcohol-induced neuroinflammation and oxidative stress; on the other, they help establish cell homeostasis following alcohol exposure [5-7]. Much research has focused on the impacts of alcohol exposure on neuronal cytotoxicity, but far fewer studies have explored the effects of alcohol on microglial integrity [8, 9]. Through the experiments performed here, we sought to investigate the impact of alcohol use on neuroinflammation and microglial toxicity.

Microglia are the brain's resident macrophage [10]. They are highly active in both the healthy and pathological central nervous system (CNS), responding to disturbances in neurons, the blood brain barrier (BBB), and blood vessels, among other constituents, to maintain CNS homeostasis [11]. It had long been assumed that microglial activation was a singular state, but more recent work has established that microglia adopt a number of phenotypes, depending on the character of the CNS challenge [1, 12]. Crucially, microglia can express both pro- and anti-inflammatory mediators in response to immune insults [13, 14]. While it is clear that acute and chronic alcohol exposure induce microglial activation, it is unknown to what degree the microglial phenotype is a cause or consequence of alcohol-induced neuroinflammation or subsequent neurodegeneration.

Alcohol induces a number of changes in the immune system, generally resulting in a pro-inflammatory state in the CNS [4, 15-18]. For example, alcohol has been shown to stimulate toll-like receptor 4 (TLR4) and toll-like receptor 2 (TLR2) expression in microglia, thereby increasing pro-inflammatory mediators, reactive oxygen species, and neuronal apoptosis [8, 9, 19]. Additionally, in a rat model of binge drinking, hippocampal microglia underwent increased activation in response to alcohol exposure [20]. However, neurotrophic microglia have also been implicated in recovery from alcohol exposure, suggesting that partial microglial activation in response to the cytotoxicity and inflammation induced by alcohol can actually remediate the negative effects of the exposure [21]. The complexity of microglial responses to alcohol necessitates further study of microglia integrity in an alcoholic brain milieu.

In 2014, our group examined the CNS gene expression profiles of rats experiencing acute alcohol exposure [22]. We identified a number of upregulated genes and transcription factors, in addition to 20 highly connected hub genes that may be involved in the immune response to acute alcohol exposure. Among these was salt-inducible kinase 1 (*SIK1*), a serine/threonine protein kinase that was originally identified in the adrenal glands of rats fed high salt diets [23]. Even though *SIK1* has largely been investigated in the extracranial context of adrenal physiology and pathology [24-26], Feldman et al. demonstrated that *SIK* mRNA is significantly increased in the rat hippocampus and cortex following kainic acid-induced seizures [27]. Since then, a number of studies have implicated *SIK1* in brain pathology [28-30], including one that identifies de novo *SIK1* mutations as the cause of certain developmental epilepsies [31]. In the present study, we have established that *SIK1* activity is crucial for regulating alcohol-induced microglial apoptosis through its effects on the NF- κ B signaling pathway.

Materials and Methods

Animals

Animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Harbin Medical University. We feed the mice as our previous work described [32]. 36 C57BL/6 mice (5 to 6 weeks old) were randomly divided into four groups. Alcohol consumption followed a two-bottle choice-drinking in the dark paradigm [33]. For 3 weeks, the animals received 0%, 5%, 10%, or 15% alcohol from separate bottles during a 3 h period starting three hours into the dark cycle to avoid side preference and were pair-fed diets with equal amounts of water. Study mice were sacrificed under anesthesia and the brain tissues were excised for analysis.

Culture of primary microglial cells

Mouse primary microglial cell cultures were prepared as previously described [22, 34]. In brief, cerebral cortices from 1-day-old neonatal mice were dissected, stripped of their meninges and vessels after washing in ice-cold PBS, and digested with 0.25% trypsin for 30 min at 37 °C. Trypsin activity was stopped with an equal volume of RPMI medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Invitrogen), 100 U/mL penicillin, and 100 lg/mL streptomycin (Invitrogen). The tissue suspension was centrifuged, supernatant removed, and the pelleted cells made into a single cell suspension in culture medium by repeated pipetting followed by passage through a 40 mL cell strainer and then centrifuged at 1250×g for 5 min at room temperature. Supernatant was discarded and the pellet was resuspended in 3 mL of 70% Percoll and transferred to a 15 mL conical tube. 6 mL of 50% Percoll followed by 2 mL of 2% FBS and 0.2% glucose in PBS were layered on top of the 70% Percoll cell suspension and centrifuged at 2400×g for 30 min at room temperature. The layer containing enriched microglia was collected from the interface between the 70% and 50% Percoll phases and washed twice with 1 mL of PBS containing 2% FBS and 0.2% glucose and centrifuged at 1250×g for 5 min at room temperature. Isolated brain microglia were seeded in growing medium (250, 000 cells/mL) and non-adherent cells were removed by washing cells with PBS 1 h after plating. Medium was replaced every 3–4 days, and cultures were used for between 12 and 20 days. For alcohol stimulation, cells were incubated with medium containing 50 mM alcohol at 37 °C, 5% CO₂ and harvested 12 h post-treatment.

RT-PCR

RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. cDNA was transcribed from 1 mg of total RNA using Reverse Transcription System (Promega, Madison, WI). SYBR-Green-based real time quantitative PCR was performed using the iCycler (Bio-Rad Laboratories Inc, Hercules, CA). Comparative threshold cycle (Ct) method was used to calculate expression relative to control groups. The final results were expressed as fold changes between the sample and the controls corrected with internal control. Sense and antisense primer sequences were: *SIK1* sense 5'- TGGACGTCTGGAGCCTCGGT-3'; *SIK1* antisense 5'- AGAGTGGGGTCTGGCCTGCAT-3'; *IL-1β* sense 5'-AATCTCACAGCAGCACATCAAG-3'; *IL-1β* antisense 5'-AGCCCATACTTTAGGAAGACA-3'; *IL-6* sense 5'-TGGAGTCACAGAAGGAGTGGCTAAG-3'; *IL-6* antisense 5'-TCTGACCACAGTGAGGAATGTCCAC-3'. *TNF-α* sense 5'-CCCCCTCAGCAAACCACCAAGT-3'; *TNF-α* antisense 5'-CTTGGGCAGATTGACCTCAGC-3'; *β-actin* sense 5'-CCACACCCGCCACCAAGTTCG-3'; *β-actin* antisense 5'-CCATTCCCACCATCACACC-3'.

Western blot

Cells or tissues were harvested and lysed with ice-cold lysis buffer (50 mM Tris-HCl, pH 6.8, 32 mM 2-ME, 2% SDS, 10% glycerol). After centrifugation (20, 000×g, 10 min, 4 °C), proteins were separated by 10% SDS PAGE and transferred to polyvinylidene difluoride membranes (Bio-rad). After blocking with 5% BSA in TBST, membranes were incubated with primary antibodies, followed by HRP-linked secondary antibodies (Santa Cruz, CA). Bound antibodies were detected by SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Antibodies against p65, Histone H1, and β-actin were purchased from Santa Cruz Biotechnologies (Santa Cruz). Anti-SIK1, anti-IKKα/β, and anti-p-IKKα/β antibodies were obtained from Abcam (Cambridge, MA) Protein levels of Histone H1 and β-actin were used as loading controls.

TUNEL Staining

TUNEL staining was performed using the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI) according to the manufacturer's protocol. Briefly, cells were grown on chambered culture slides, fixed with 4% PFA for 2 h at room temperature, and permeabilized with 0.2% Triton X-100 in PBS for 5 min. Cells were washed again with PBS and equilibrated with 100 μ L of equilibration buffer at room temperature for 10 min. Slides were covered with 50 μ L of terminal deoxynucleotidyl transferase reaction mixture for 60 min in a humidified chamber. The reaction was stopped with 2x SSC for 15 min. Nuclei were visualized by DAPI staining.

Caspase Activity Assays

Microglial cells (1×10^4) were incubated in 96-well plates for 24 h, and caspase-3 activity was measured using the caspase-3 and caspase-9 assay kits (Ambion, Austin, TX) according to the manufacturer's protocol. Briefly, the cells were incubated with lysis buffer on ice for 10 min and collected via 10,000 $\times$ g centrifugation for 10 min. Protein (100 μ g) from each sample was incubated with specific colorimetric tetrapeptides Asp-Glu-Val-Asp-p-nitroaniline (pNA; specific substrate of caspase-3) or Leu-Glu-His-Asp-pNA (specific substrate of caspase-9) at 37°C for 60 min. The activity levels of caspase-3 and caspase-9 were quantified using a spectrophotometer at 405 nm, and the data were normalized to the control group.

Immunofluorescent staining

Microglial cells were seeded in 4-well chamber slides in triplicate. Cells were then fixed with freshly prepared 4% PFA for 15 min and permeabilized with 0.5% Triton X-100 for 15 min. After 1 h incubation with 10% normal goat serum, cells were incubated with rabbit anti-p65 (1:100, Santa Cruz). After washing with PBS, a FITC-conjugated goat anti-rabbit secondary antibody (1:1000, Invitrogen) was used to probe the primary antibody for 1 h. Thereafter, the cells were mounted with DAPI containing mount media and scanned under an Olympus fluorescence microscope. Cells probed with only a secondary antibody were used as negative controls.

Statistical analysis

Data were expressed as mean $\pm$ SD from at least three separate experiments. Differences between groups were analyzed using one-way ANOVA. $p < 0.05$ was considered statistically significant.

Results

SIK1 is increased in alcohol-treated microglial cells and brain tissues

From our previous microarray data, we found that *SIK1* is induced by alcohol in rodent brain tissue [22]. To investigate whether SIK1 plays a role in alcohol-induced rodent brain apoptosis and inflammation, we performed RT-PCR to

Fig. 1. SIK1 is increased in alcohol-treated microglial cells and brain tissues. (A) RT-PCR analysis of SIK1 mRNA expression in the brain of mice fed with alcohol (0%, 5%, 10% or 15%) for 3 weeks. (B) Western blot analysis of SIK1 protein expression in the brain of mice fed with alcohol (0%, 5%, 10% or 15%) for 3 weeks. (C) Tissue fluorescence staining of SIK1 in the brains of mice fed with 0% alcohol (-) or 15% alcohol (+) for 3 weeks. (D) RT-PCR analysis of SIK1 mRNA expression in mouse microglial cells treated with alcohol (100 mM). (E) Western blot analysis of SIK1 protein expression in mouse microglial cells treated with alcohol (100 mM). * $p < 0.01$ compared with the control group.

analyze SIK1 mRNA and protein expression in the brain of mice fed with different concentrations of alcohol. We found that SIK1 is increased in mouse brains after alcohol consumption (Figs. 1A and 1B). Brain tissue fluorescence staining of SIK1 was then performed in mice fed with or without alcohol (15%). SIK1 staining in the alcohol group was stronger than in the control group in both microglial cells and neurons (Fig. 1C). We focused on microglial cells mediated brain inflammation. We isolated mouse primary microglial cells from newborn mice and treated the cells with

100 mM alcohol. We then performed RT-PCR and western blots to analyze *SIK1* expression 12 h post-treatment. We found that expression of *SIK1* was significantly higher after alcohol treatment compared with control (Fig. 1D and 1E). Together, these results indicate that alcohol-treated microglia have increased expression levels of *SIK1*.

Knockdown of *SIK1* increases alcohol-induced apoptosis in microglial cells

To explore the role of *SIK1* in alcohol-induced microglial cell apoptosis, we first transfected mouse microglial cells with control or *SIK1* siRNA for 24 h and exposed the cells to alcohol (100mM). Knockdown efficiency was confirmed by western blotting (Fig. 2A). As expected, cell viability decreased by 30% in the alcohol-treated as compared untreated cells. However, the viability of *SIK1*-knockdown cells decreased by almost 50% with alcohol treatment (Fig. 2B). TUNEL staining, caspase-3 and caspase-9 activity assays and consistently showed that alcohol-induced cell apoptosis was promoted by depletion of *SIK1* (Fig. 2C to 2E). Overall, these results suggest that *SIK1* is protective against alcohol-induced apoptosis in microglia.

SIK1 regulates NF- κ B signaling activity

To investigate whether *SIK1* regulates alcohol-induced rodent brain inflammation, RT-PCR analysis was used to evaluate expression of inflammatory factors in *SIK1* knockdown microglial cells. *IL-1 β* , *IL-6*, and *TNF- α* expression was stimulated by alcohol and further promoted by *SIK1* knockdown (Figs. 3A to 3C). Pearson correlation analysis showed a negative correlation between *SIK1* and inflammatory factors (*IL-1 β* , *IL-6*, *TNF- α*) in the brain from nine mice fed with alcohol (Figs. 3D to 3F). To study whether NF- κ B signaling is related to *SIK1*-mediated inflammation induced by alcohol, we treated mouse microglial cells with alcohol after *SIK1* knockdown and extracted nuclear proteins for p65 expression. Alcohol promoted p65 nuclear translocation, which was enhanced by *SIK1* knockdown (Fig. 4A). Cell fluorescence staining and NF- κ B luciferase activity assays confirmed that knockdown of *SIK1* in microglial cells further promoted NF- κ B activity induced by alcohol (Figs. 4B and 4C). Mechanistically, knockdown of *SIK1* elevated phosphorylated IKK α / β and subsequently stimulated NF- κ B signaling activity (Fig. 4D).

Fig. 2. Knockdown of *SIK1* increased alcohol-induced apoptosis in microglia. (A) *SIK1* expression measured by western blotting after *SIK1* siRNA transfection. (B to E) Cell viability assay (B), TUNEL staining (C), caspase-3 (D), and caspase-9 (E) activity assays in *SIK1*-knockdown microglia treated or not treated with alcohol (100mM). *p<0.01 compared with the control group not treated with alcohol; &p<0.01 compared with the control group treated with alcohol.

Fig. 3. There is a negative correlation between SIK1 expression and pro-inflammatory cytokines in alcohol-induced microglia and brain tissues. (A to C) RT-PCR analysis of IL-1 β (A), IL-6 (B), and TNF- α (C) mRNA expression in mouse microglia treated or not treated with alcohol (100mM) after SIK1 siRNA transfection. (D to F) Pearson correlation analysis between IL-1 β and SIK1 (D), IL-6 and SIK1 (E), and TNF- α and SIK1 (F). * $p < 0.01$ compared with the control group not treated with alcohol; & $p < 0.01$ compared with the control group treated with alcohol.

Fig. 4. Knockdown of SIK1 promotes NF- κ B signaling activity. (A to D) Western blot analysis of nuclear p65 protein levels (A), Cell fluorescence staining of p65 (B), Relative NF- κ B luciferase activity (C), Western blot analysis of IKK α / β and p-IKK α / β in mouse microglia treated or not treated with alcohol (50mM) after SIK1 siRNA transfection (D). * $p < 0.01$ compared with the control group not treated with alcohol; & $p < 0.01$ compared with the control group treated with alcohol.

NF- κ B signaling plays a role in alcohol-induced apoptosis of microglial cells

In order to detect the role of p65 in alcohol-induced microglial cell apoptosis, we first transfected mouse microglial cells with control or p65 siRNA for 24 h and exposed the cells

to alcohol (100mM). Knockdown efficiency was confirmed by western blotting (Fig. 5A). We performed cell viability assays and found that cell viability decreased by 40% in the alcohol-treated as compared to cells in the control group. However, the viability of p65-knockdown cells decreased by only 20% with alcohol treatment (Fig. 5B). TUNEL staining, caspase-3, and caspase-9 activity assays consistently revealed that alcohol-induced cell apoptosis was inhibited by depletion of p65 (Figs. 5C to 5E). Together, these findings indicate that NF- κ B signaling promotes alcohol-induced microglial apoptosis.

NF- κ B signaling is required for SIK1-mediated apoptosis induced by alcohol in microglial cells

We next investigated whether SIK1 regulates alcohol-induced inflammation through the NF- κ B signaling pathway. SIK1 was further decreased in p65 knockdown microglial cells. These cells were subjected to cell viability, TUNEL staining, caspase-3 and caspase-9 activity assays (Figs. 6A to 6D). As compared to p65 knockdown cells, further downregulation of SIK1 did not affect microglial cell apoptosis. These results strongly suggest that NF- κ B signaling is required for SIK1-mediated apoptosis induced by alcohol.

Discussion

Despite being present in almost all societies throughout history, alcohol consumption continues to contribute significantly to the global burden of disease [35]. Acute alcohol

Fig. 5. NF- κ B signaling plays a role in alcohol-induced apoptosis of microglia. (A) p65 expression measured by western blotting after p65 siRNA transfection. (B to E) Cell viability assay (B), TUNEL staining (C), caspase-3 (D), and caspase-9 (E) activity assays in p65-knockdown microglia treated or not treated with alcohol (100mM). *p<0.01 compared with the control group not treated with alcohol; &p<0.01 compared with the control group treated with alcohol.

Fig. 6. NF- κ B signaling is required for SIK1-mediated apoptosis induced by alcohol in microglia. (A to D) Cell viability assay (A), TUNEL staining (B), caspase-3 (C), and caspase-9 (D) activity assays in p65-, SIK1-, or both p65- and SIK1- knockdown microglia treated or not treated with alcohol (100mM). *p<0.01 compared with the control group not treated with alcohol; &p<0.01 compared with the control group treated with alcohol.

intoxication is a risk factor for myriad clinical conditions, including brain damage, white matter atrophy, and eventual neurodegeneration [36, 37]. One consequence of alcohol consumption that likely contributes to its adverse effects is alcohol-induced neuroinflammation. Alcohol generates a pro-inflammatory environment both extracranially and intracranially, where there is a relatively long-lasting increase in pro-inflammatory cytokines (TNF α , IL-1 β , and MCP-1) [38, 39]. The brains of human alcoholics were shown to have increases in both MCP-1 and microglial activation, along with increased microglial expression of Iba-1 and/or GluT₅ [17]. Yet, apart from their potential role in alcohol-induced cytotoxicity and neuroinflammation, microglia also likely help restore cell homeostasis following alcohol exposure [1, 13]. In this study, we have provided insight into how the gene *SIK1* regulates the NF- κ B signaling pathway, ultimately to reduce alcohol-induced microglial apoptosis.

While there is much research into alcohol-induced neuronal toxicity, relatively little is known about the effects of alcohol consumption on microglial integrity [40]. Microglia are highly responsive to neurodegenerative insults and adopt a number of phenotypes, depending on the type of CNS challenge [41]. In an inflammatory state, such as that generated by alcohol consumption, microglia contribute to the production of reactive oxygen species, which have neurotoxic effects, including to oligodendrocytes, whose loss contributes to certain neurodegenerative diseases [2, 41]. Microglia, as the primary immune effectors of the CNS, function similarly to macrophages in that they are antigen presenting cells (APCs) and travel to the site of tissue damage to secrete cytokines and other pro-inflammatory factors [42, 43]. However, microglia also have a strong neuroprotective effect, which can heighten in a neurotoxic milieu [44]. For example, microglia have been shown to effectively scavenge oxygen-derived free radicals during excitotoxin-induced neurodegeneration [45]. Researchers have also demonstrated not only that microglia contribute to maintaining neural homeostasis, but also that they promote beneficial circuit-level alterations such as synaptic plasticity through brain-derived neurotrophic factor (BDNF) signaling [46]. Microglia similarly increase trophin-mediated healing responses following spinal cord injury [47]. That we found a significant increase in alcohol-induced microglial apoptosis in our rodent model of acute alcohol use suggests the short- and long-term neurotoxic effects of alcohol use may be due in part to reductions in the reparative activity of microglia. Interestingly, alcohol could reduced Inflammatory Response in Human Lung Epithelial Cells [48].

We began exploring the cause of alcohol-induced microglial apoptosis by examining the expression levels of *SIK1*, which we had previously shown to be upregulated in the brains of rats experiencing acute alcohol exposure [22]. Indeed, in the present study we found that expression of *SIK1* was significantly higher after alcohol treatment compared with control. Using TUNEL staining, caspase-3, and caspase-9 activity assays, we then demonstrated that *SIK1* depletion promoted alcohol-induced microglial apoptosis—suggesting that *SIK1* exerts a protective effect on microglia in the context of acute alcohol consumption. We then found that expression of inflammatory mediators IL-1 β , IL-6, and TNF- α was stimulated by alcohol and further promoted by *SIK1* knockdown. Furthermore, cell fluorescence staining and NF- κ B luciferase activity assays indicated that *SIK1* knockdown in microglia promoted alcohol-induced NF- κ B activity. Ultimately, we established through the use of p65 knockdown cells that NF- κ B activity is in fact necessary for *SIK1*-mediated, alcohol-induced microglial apoptosis. *SIK1* has been reported to interacts with TGF- β -activated kinase 1-binding protein 2 (TAB2) and interrupt the complex of TAB2-TNF receptor-associated factor 6 (TRAF6). Thereby inhibits TLR4-mediated NF- κ B signaling [49]. Microglia are the primary source of the pro-inflammatory cytokine and contributed to neuroinflammation [50, 51]. Though we found expression of *Sik1* in both microglia and neuron, we focused on microglia-mediated neuroinflammation in this study. The role of *Sik1* in neuronal development needs further investigation.

The NF- κ B signaling pathway contributes to alcohol-induced neuroinflammation [52]. In fact, the anti-inflammatory xanthohumol (XN) has been shown to inhibit microglial inflammatory responses, in part through reducing NF- κ B signaling activation [53]. While it is clear that the NF- κ B signaling pathway is implicated in both alcohol-induced

neuroinflammation and microglial activation, it is less known whether its activity is destructive or restorative. In the present study, we have shed some light on this question by establishing that NF- κ B signaling is necessary for *SIK1*-mediated, alcohol induced microglial apoptosis—a response that likely impedes the neurotrophic functions of microglia following alcohol exposure. However, longer term studies are needed to determine to what degree alcohol-induced microglial apoptosis contributes to the exacerbation or prevention of alcohol use-related neurodegeneration.

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Disclosure Statement

No conflict of interests exists.

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